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14. ABSTRACT

The role of innate immunity in prostate cancer tumorigenesis is unclear. We hypothesis that innate immune pathways contribute to programming the inflammatory component of the tumor microenvironment and that activation of these pathways may selectively skew this immune composition and alter tumor growth. Pattern recognition receptors such as Toll-like receptors (TLRs) are key signaling molecules that regulate innate and adaptive immune responses in the presence of pathogens and endogenous ligands. We have generated and characterized TRAMP Tg$^{+/-}$ x MyD88$^{-/-}$ mice. We showed that *de novo* prostate cancers in absence of MyD88 develop higher grade adenocarcinomas than wild-type controls at 30 weeks of age. Analysis of tumor infiltrating cells revealed increased infiltration of macrophage lineage cells, characterized as myeloid-derived suppressor cells (MDSCs), and decreased CD8 T lymphocytes and NK cells. We have shown that MyD88 plays in intrinsic role in the differentiation of MDSCs, with the absence of MyD88 biasing development towards the granulocytic subtype. MyD88-deficient MDSCs have an increased migration in response to the endogenous ligand S100A9, suggesting a role of MyD88 in governing MDSC homeostasis that can be leveraged as an anti-tumor therapy.

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Prostate cancer, tumor microenvironment, Toll-like receptors

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Introduction

Prostate cancer is the most prevalent non-skin solid malignancy and the second-leading cause of cancer-related mortality in men in the U.S.[1]. Treatment of metastatic prostate cancer with androgen-deprivation therapy ultimately leads to development of castration-resistant disease, where cancer cells become more responsive to even minute quantities of testosterone. Promising therapies are available for castration-resistant prostate cancer (CRPC), including chemotherapy, immune-based therapies, therapies targeting bone metastasis, and second line hormone therapies, however, all with a finite efficacy. Improved and likely combinatorial therapies will be necessary.

Inflammation has long been associated with the prostate cancer microenvironment, and may facilitate tumor growth or promote an anti-tumor immune response. Evidence suggests that cancer cells can be hijacking inflammatory pathways to promote angiogenesis and proliferation[2]. Conversely, inflammation can trigger the infiltration of cytotoxic immune effector cells, resulting in the production of clonal $CD8^+$ T cells[3]. However, the contribution of the tumor infiltrating lymphocytes (TILs) to prostate cancer development, growth, and metastasis is unclear. We are interested in understanding the mechanisms for development of TILs and how they modulate prostate cancer. Our hypothesis is that the innate immune response can program TILs and play a key role in tumor surveillance, are important in generation of tumor-specific immunity, and that by tumor growth can be altered through modulating the composition of TILs through innate immunity.

Body

Pathogens or cancerous cells alike can produce danger signals that elicit the activation of immune responses. These signals in the form of conserved molecules termed pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) can be discriminated from self-antigens by a family of pattern-recognition receptors of innate immunity, including Toll-like receptors (TLRs). Thirteen mammalian TLRs have been identified to date with ligands ranging from lipopolysaccharide (LPS) found in gram-negative bacterial walls recognized by TLR4, double stranded RNA produced by many viruses for TLR3, viral CpG motifs with TLR9, to endogenous ligands such as heat-shock protein 70 and chromatin component HMG-B1. Activation of these receptors leads to induction of multiple inflammatory pathways, including nuclear factor-kappa B (NF-κB) and interferon regulatory factors (IRFs), which may mediate the development of cytotoxic T lymphocytes (CTLs) and dendritic cell (DC) maturation[4]. Although TLRs have been shown to inhibit negative regulatory cells such as Tregs, the relationship between TLRs and myeloid-derived suppressor cells (MDSCs) is less clear[4-5].

TLRs recruit adaptor proteins such as MyD88 and serine kinase IL-1 receptor-associated kinase (IRAK), leading to activation of MAP kinases, NF-κB, and expression of inflammatory genes. Most TLRs utilize the MyD88 pathway. The role of TLRs in modulating cancer is conflicting, as prior reports have suggested tumor promoting as well

as suppressing effects. Deficiency in MyD88 confers decreased development of tumors in a mouse model of spontaneous intestinal tumorigenesis and diethylnitrosamine-induced hepatocellular tumors[6-7]. In contrast, a recent report suggested that MyD88 inhibition promoted pancreatic cancer growth through dendritic cell and Th2 activation[8].

We have been focused on studying the phenotype of TRAMP Tg^{+/-} x MyD88^{-/-} mice, work described in **Specific Aim 2**. This has culminated in a publication now e-published and in the April 2015 Edition of *The Prostate,* entitled "Loss of MyD88 Leads to More Aggressive TRAMP Prostate Cancer and Influences Tumor Infiltrating Lymphocytes". In summary, we showed that the absence of MyD88 led to increased prostatic intraepithelial neoplasm (PIN) and areas of well-differentiated adenocarcinoma in TRAMP transgenic mice. Analysis of infiltrating immune populations revealed an increase in CD11b^{+} cells and a deficiency in NK cells in prostates from MyD88^{-/-} TRAMP[Tg+/-] compared to MyD88^{+/+} TRAMP[Tg+/-] mice, whereas a decrease in splenocytic NK cell differentiation was observed in MyD88^{-/-} mice. Prostate tumors revealed no significant differences in NF-κB or AR expression in MyD88^{+/+} TRAMP[Tg+/-] compared to MyD88^{-/-} TRAMP[Tg+/-] mice.

In our 2014 Annual Summary, we presented limitations to our initial aims using TRAMP Tg^{+/-} animals, namely the length of time for development of tumors from 24 to 30 weeks of age, the ubiquitous presence of our gene knockout in prostate epithelium, stroma, as well as immune system, and the fixed nature of the prostate model with expression of the large T antigen, which may have limited translational implications. We proposed a model of disease progression in prostate cancer, where damage-associated molecular patterns (DAMPs) released by the tumor stimulate the innate immune pathways through pattern recognition receptors (PRRs) including the TLRs and intracellular Nod-like receptors (NLRs). To parse out the role of TLR signaling in various compartments, we proposed adapting a previously published subcutaneous prostate tumor model based on lentiviral transfection of primary prostate epithelium[11-12]. This model has been developed in Owen Witte's laboratory at UCLA, which we are collaborating with. Prior reports have been performed using both human and murine prostate epithelium on an immunocompromised background. We tested a syngenic immunocompotent model using murine prostate epithelium on a C57Bl6 host which led to exciting preliminary data showing that just the presence of an intact immune system altered tumor growth with larger tumors in a C57Bl6 versus SCID background (**Fig 1**). The flexibility of the model allows variation in the oncogenic drivers of the tumors, which subsequently produces disease ranging from PIN (AKT/ERG and TRAMP) to castration-resistant prostate cancer (AKT/ERG + AR). As the tumor cells are combined with fetal mesenchymal cells for implantation, this model allows for the genotypic manipulation of both the tumor and its surrounding stroma. We presented preliminary data using this system in our 2015 Annual Summary. We encountered complications with poor lentiviral infection efficiency and expression of AKT. During this time, we continued studying the mechanisms of increased CD11b^{+} cells in MyD88^{-/-} TRAMP[Tg+/-] compared to MyD88^{+/+} TRAMP[Tg+/-] mice, which we presented in our 2016 Annual Summary.
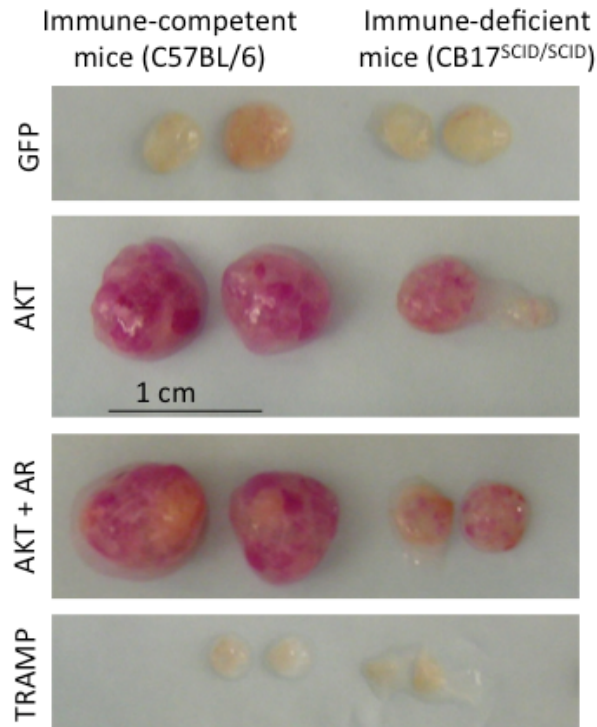
Figure 1. Larger C57Bl6 tumors generated by retroviral infection by AKT and AKT + AR in a C57Bl6 versus CB17scid/scid background.

We performed further staining of infiltrating immune populations in tumors from 30 week-old MyD88$^{+/+}$ TRAMP$^{Tg+/-}$ and MyD88$^{-/-}$ TRAMP$^{Tg+/-}$ mice, which revealed an increase in CD11b^{+}Gr1^{+} cells suggestive of myeloid-derived suppressor cells (MDSCs). Analysis of tumors by qPCR revealed an increase in iNOS and l-arginine, which mediate the T cell inhibitory function of MDSCs. We began examining differences in various cytokines and chemokines important in MDSC development and recruitment and found increased expression of multiple chemokines in MyD88$^{-/-}$ TRAMP$^{Tg+/-}$ mice such as IL-1, IL-13, CCL-2, CCL-3, CCL-5, and CXCL-1 (**Fig. 2**) that is updated in our current and final 2017 Annual Summary.
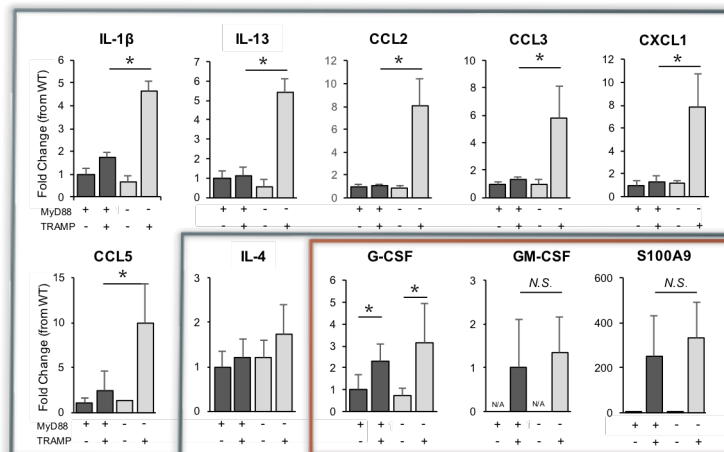

Figure 2. Expression of cytokines and chemokines as listed were examined by qPCR from fresh frozen tumors derived from 30 week-old MyD88$^{+/+}$ TRAMP$^{Tg+/-}$ and MyD88$^{-/-}$ TRAMP$^{Tg+/-}$ mice.

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To further examine the potential mechanisms and to determine if there is an intrinsic role of MyD88 to develop myeloid subtypes, we optimized and performed *in vitro* differentiation of bone marrow to various myeloid subtypes using combinations of GM-CSF, G-CSF, and M-CSF (**Fig. 3**). We found that MyD88-dependent signaling pathways are important in the homeostasis of T cell inhibitory granulocytic MDSCs as MyD88$^{-/-}$ bone marrow preferentially developed CD11b^{+}Ly6G^{hi}Ly6C^{lo} granulocytic MDSCs compared to CD11b^{+}Ly6G^{lo}Ly6C^{hi} monocytic MDSCs. Using a transwell migration assay, we have shown that bone marrow derived MyD88$^{-/-}$ CD11b^{+}Ly6G^{hi}Ly6C^{lo} granulocytic MDSCs have increased migration in response to the endogenous inflammatory protein SA100A9, but not HMGB1 and exogenous ligands polyIC and CpG compared to MyD88$^{+/+}$ CD11b^{+}Ly6G^{hi}Ly6C^{lo} granulocytic MDSCs (**Fig. 4**).

Figure 3. Increased granulocytic CD11b^{+}Ly6G^{hi}Ly6C^{lo} MDSCs from *in vitro* differentiated bone marrow derived from MyD88$^{-/-}$ compared to MyD88$^{+/+}$ mice.
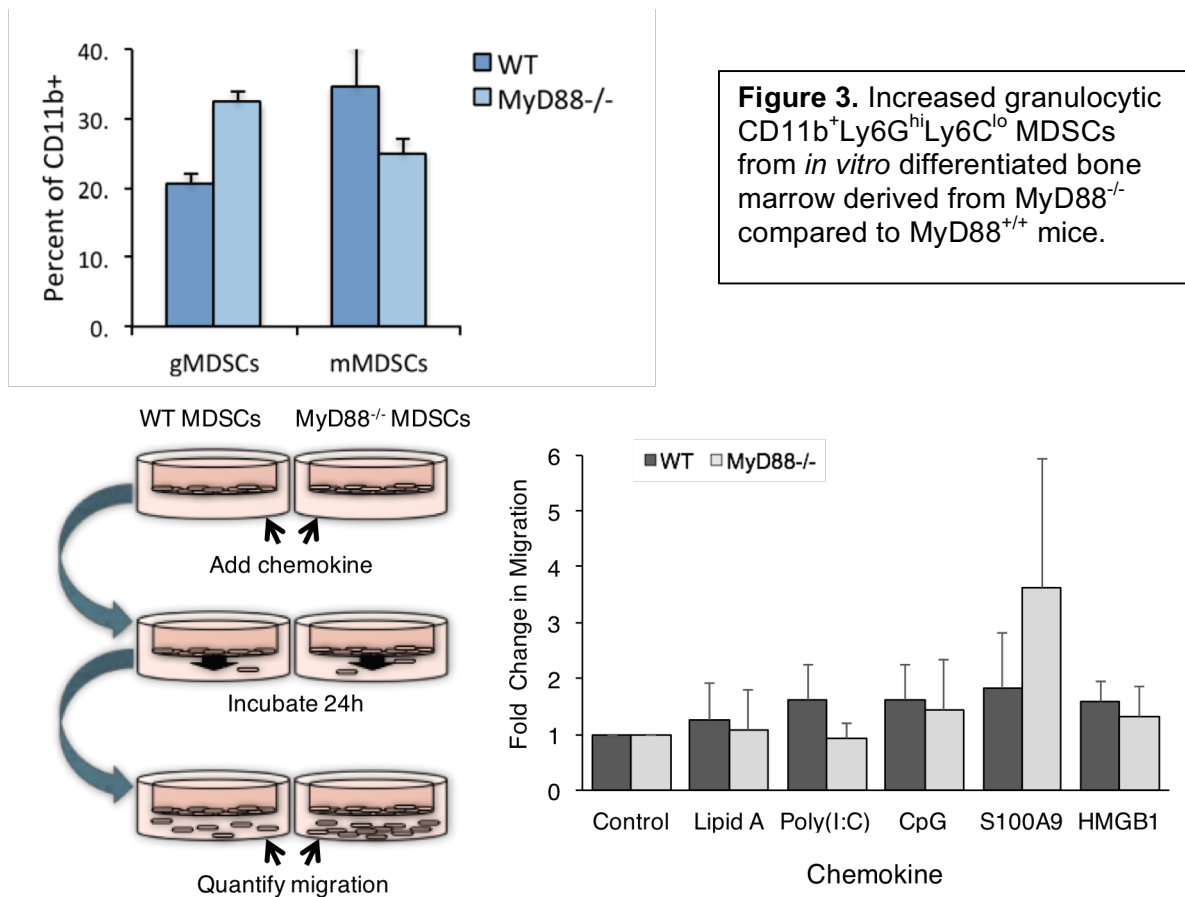

Figure 4. Increased migration of CD11b^{+}Ly6G^{hi}Ly6C^{lo} MDSCs in response to S100A9 from *in vitro* differentiated bone marrow derived from MyD88$^{-/-}$ compared to MyD88$^{+/+}$ mice.

This work is building on our ongoing model suggesting that MyD88 functions as a negative regulator of MDSC differentiation to the granulocytic subtype and promoting anti-tumor immunity (**Fig. 5**). We propose that activation of TLR signaling through MyD88-dependent pathways may induce an anti-tumor program through suppression of negative T cell regulators by inhibiting MDSC development and migration, and may be an adjunct in the treatment of prostate cancer. We hypothesis that S100A9 may recruit MDSCs to tumors through a MyD88-dependent manner and are currently investigating pre-clinical and clinical evidence of using inhibitors of S100A9, such as Tasquinimod, in conjunction with checkpoint inhibitors that augment T cell mediated anti-tumor immunity in prostate cancer. A similar concept was recently described, which validates our hypothesis[13].
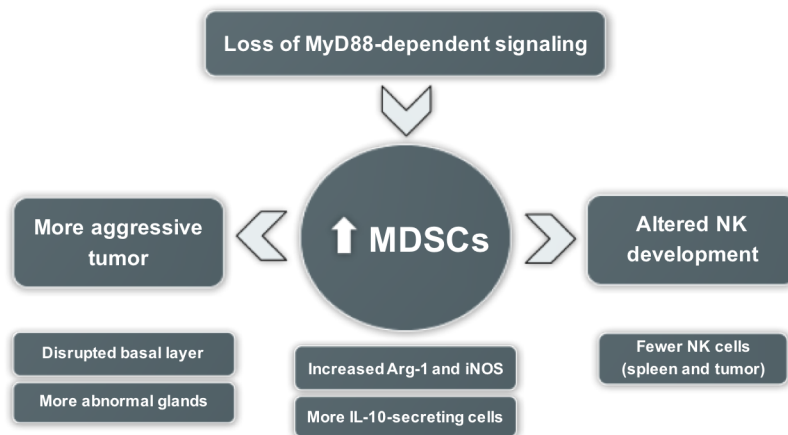

Figure 5. Summary of our MyD88-deficient TRAMP model of prostate cancer.

Key Research Accomplishments

- We have published our manuscript describing the role of MyD88 in prostate cancer tumorigenesis and composition of the immune microenvironment in *The Prostate*
- We have shown an intrinsic increase in granulocytic $CD11b^+Ly6G^{hi}Ly6C^{lo}$ MDSC development from bone marrow-derived *in vitro* differentiated cells
- We have shown that $MyD88^{-/-}$ granulocytic $CD11b^+Ly6G^{hi}Ly6C^{lo}$ MDSCs have increased migration in response to stimuli by S100A9, but not HMGB1, CpG, and polyIC compared to the wild-type counterpart.
- We are investigating the pre-clinical and clinical role of S100A9 inhibition in the combinatorial therapy of advanced prostate cancer with checkpoint inhibitors.

Reportable Outcomes

We have presented this work in yearly seminars at the UCLA Prostate SPORE Lecture Series as well as the manuscript published in *The Prostate.* Much of this work was performed by Elizabeth Peek, a PhD candidate that successfully defended her thesis in the end of 2016.

Conclusions

We have shown that TRAMP Tg$^{+/-}$ x MyD88-deficient mice result in accelerated prostate cancer development with increased infiltration of immature CD11b$^+$Gr1$^+$ myeloid cells and decreased T lymphocytes. We have shown a MyD88-dependent intrinsic ability to skew the differentiation between granulocytic and monocytic MDSCs and to migration in response to S100A9. We propose that activation of TLR signaling through MyD88-dependent pathways may induce an anti-tumor program through suppression of negative T cell regulators by inhibiting MDSC development and migration, and may be an adjunct in the treatment of prostate cancer in conjunction with checkpoint inhibitors.

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Loss of MyD88 Leads to More Aggressive TRAMP Prostate Cancer and Influences Tumor Infiltrating Lymphocytes

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BACKGROUND. The influence of pattern recognition receptor (PRR) signaling in the prostate tumor microenvironment remains unclear. Although there may be a role for PRR agonists as adjuvants to therapy, prior evidence suggests tumor promoting as well as tumor inhibiting mechanisms. The purpose of this study is to examine the role of the key Toll-like receptor (TLR) signaling adaptor protein myeloid differentiation primary response gene 88 (MyD88) in prostate cancer development.

METHODS. MyD88$^{-/-}$ mice in a C57Bl6 background were crossed with transgenic adenocarcinomas of the mouse prostate (TRAMP) mice to create MyD88$^{-/-}$ TRAMP$^{Tg+/-}$ animals, which were compared to MyD88$^{+/+}$ TRAMP$^{Tg+/-}$ animals and their non-transgenic counterparts at 30 weeks. Prostates were examined histologically, by immunohistochemistry and immunofluorescence staining, and by qPCR, to characterize tumor-infiltrating immune populations as well as activation of the downstream NF-κB pathway and androgen receptor (AR) expression. Splenocytes were examined for development of distinct immune cell populations.

RESULTS. Absence of MyD88 led to increased prostatic intraepithelial neoplasm (PIN) and areas of well-differentiated adenocarcinoma in TRAMP transgenic mice. Analysis of infiltrating immune populations revealed an increase in CD11b$^+$ Gr1$^+$ myeloid-derived suppressor cells (MDSCs), as evidenced by increased expression of prostatic arginase-1 and iNOS as well as the cytokine IL-10, and a deficiency in NK cells in prostates from MyD88$^{-/-}$ TRAMP$^{Tg+/-}$ compared to MyD88$^{+/+}$ TRAMP$^{Tg+/-}$ mice, whereas a decrease in splenocytic NK cell differentiation was observed in MyD88$^{-/-}$ mice. Prostate tumors revealed no significant differences in NF-κB or AR expression in MyD88$^{+/+}$ TRAMP$^{Tg+/-}$ compared to MyD88$^{-/-}$ TRAMP$^{Tg+/-}$ mice.

CONCLUSIONS. During prostate cancer development in the TRAMP model, MyD88 may play a role in limiting prostate tumorigenesis by altering tumor-infiltrating immune populations. This suggests that in the context of specific cancers, distinct PRRs and signaling

pathways of innate immune signaling may influence the tumor microenvironment and represent a novel therapeutic strategy. *Prostate*
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INTRODUCTION

Inflammation within the prostate cancer microenvironment is often observed adjacent to areas of focal atrophy and adenocarcinoma, although the contribution of distinct subsets of tumor infiltrating lymphocytes (TILs) to prostate cancer development, growth, and metastasis is unclear [1]. These inflammatory processes may promote anti-tumor responses, as clonal expansion and presence of circulating prostate-antigen specific CD8^{+} T cells have been observed clinically [2]. Conversely, pro-tumor inflammation has been observed with the release of pro-inflammatory chemotactic agents from areas of tumor necrosis into the tumor microenvironment that stimulate angiogenesis and proliferation [3–5]. The prostate cancer microenvironment may be globally immunosuppressive, as studies have linked TGF-β production to a bias of CD4^{+} T cells in the human prostate cancer microenvironment towards both CD4^{+}CD25^{+}Foxp3^{+} regulatory T cells (T_{regs}) and Th17 cells [6,7]. Nonetheless, the clinical importance of the immune system in prostate cancer is borne out by the efficacy of the cancer vaccine sipuleucel-T, which justifies the necessity to parse out the contributions of distinct inflammatory pathways and to examine for adjuvants to tumor immunity.

Pathogens or cancerous cells alike can produce danger signals that elicit the activation of immune responses. These signals, consisting of conserved molecules termed pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs), can be discriminated from self-antigens by a family of pattern-recognition receptors (PRRs) such as the Toll-like receptors (TLRs) of the innate immune system [8–12]. Thirteen mammalian TLRs have been identified to date with ligands ranging from lipopolysaccharide (LPS) found in gram-negative bacterial walls recognized by TLR4, double stranded RNA produced by viruses recognized by TLR3, viral CpG motifs by TLR9, to endogenous ligands, such as heat-shock protein 70 and chromatin component HMG-B1 [8,13]. TLRs recruit adaptor proteins that promote activation of downstream transcription factors such as NF-κB or interferon regulator factors (IRFs), mediating downstream development of adaptive immune effector cells such as cytotoxic T lymphocytes (CTLs) and dendritic cell (DC) maturation [14].

Although the majority of TLRs recruit the adaptor protein myeloid differentiation primary response gene 88 (MyD88), TLR3 exclusively interacts with the adaptor protein TIR-domain-containing adapter-inducing interferon-β (TRIF) to activate a MyD88-independent pathway leading to IRF3 activation and production of type I interferons. We have previously implicated the pattern recognition receptor TLR3 and type I interferons to play a critical role in prostate cancer immune surveillance in TRAMP mice, with increased tumor growth in absence of TLR3 [15]. Using polyI:C as a TLR3 agonist, we showed a marked reduction in prostate cancer growth which influenced the tumor microenvironment by creating an influx of CD3^{+} T cells and NK cells [15]. The role of TLR signaling in the inherent development of prostate cancer has important clinical correlation, as sequence variants in a 3'-untranslated region of TLR4 and polymorphisms in the TLR gene cluster encoding TLR1, 6, 10, and the downstream signaling mediators IRAK1 and IRAK4, confer increased prostate cancer risk [16–19]. How distinct TLR signaling pathways modulate the prostate cancer tumor immune environment is an open question.

In this study, we investigated the role of MyD88 in prostate cancer development using the autochthonous TRAMP model. TRAMP mice express the SV40 large T antigen in the prostate epithelium under the control of the rat probasin promoter, and are a well-described immunocompetent prostate cancer model that develops histologic PIN by 8 to 12 weeks of age and adenocarcinoma by 24 to 30 weeks of age. We hypothesized that loss of MyD88 will promote prostate cancer development, as a result of alterations in tumor-infiltrating immune populations. This work complements our prior studies of TLR3 in prostate cancer and the intracellular Nod-like receptor pathway in bladder cancer, to extend the idea that distinct PRRs differentially mediate tumor immune surveillance [15,20].

MATERIALS AND METHODS

Mice

TRAMP[Tg+/−] mice (Jackson Laboratories) on a C57Bl/6 background were genotyped as previously

described [21,22]. MyD88$^{-/-}$ mice backcrossed to a C57Bl/6 background for 10 generations were bred with TRAMP transgenic mice to homozygosity generating MyD88$^{-/-}$ TRAMP$^{Tg-/-}$ and MyD88$^{-/-}$ TRAMP$^{Tg+/-}$ mice [23]. Mice were housed in pathogen-free conditions in accordance to UCLA Animal Research Committee protocols. All animal work was performed through the approved UCLA Institutional Animal Care and Use Committee protocol #2010–023-11C in accordance with the Public Health Service Policy on Human Care and Use of Laboratory Animals and USDA Animal Welfare Act Regulations.

Tumor Models

MyD88$^{+/+}$ TRAMP$^{Tg-/-}$, MyD88$^{+/+}$ TRAMP$^{Tg+/-}$, MyD88$^{-/-}$ TRAMP$^{Tg-/-}$, and MyD88$^{-/-}$ TRAMP$^{Tg+/-}$ male mice at 25 and 30 weeks of age were sacrificed, with lungs, liver, and abdominal lymph nodes grossly inspected for metastases. Whole prostates with seminal vesicles were removed, weighed, and a portion fixed in formalin or embedded in OCT. Spleens were removed and dispersed into single cell suspensions for flow cytometric analysis of immune populations.

Histology

Representative paraffin embedded, formalin-fixed tissues were sectioned at $0.4\,\mu m$ and stained by hematoxylin and eosin. Images were assessed by light microscopy using an Axio Imager 2 (Zeiss).

Immunofluorescence and Immunohistochemistry

Immunofluorescence was performed on OCT-embedded tissue. Sections were fixed in 4% paraformaldehyde for 10 min and then blocked for 1 hr with either standard (5% BSA and 5% goat serum in PBS) or specific, when using mouse primary antibodies, (M.O.M kit block, Vector Labs) reagents. Sections were stained overnight at 4°C with anti-CD8 at 1:100 (53-6.7, R&D Systems), anti-CD11b at 1:400 (M1/70, R&D Systems), anti-Gr-1 at 1:300 (RB6-8C5, eBioscience), anti-CD49 at 1:300 (DX5, Biolegend), anti-Foxp3 at 1:300 (MF23, BD Biosciences), and anti-AR at 1:2000 (ab3510, Abcam). Secondary antibodies using goat anti-rat Al488 (Invitrogen) or goat anti-rabbit Al594 (Invitrogen) were incubated at 1:750 for 1 hr and sections counterstained with DAPI and mounted using Vectorshield (Vector Labs). Images were assessed by fluorescence microscopy using an Axio Imager 2 (Zeiss).

Immunohistochemistry was performed on formalin-fixed and paraffin embedded tissues. Sections were deparaffinized and rehydrated before blocking for one hour in 5% BSA and 5% goat serum in PBS. Sections were stained with anti-p40 at 1:50 (2073, BioSB), anti-MyD88 1:100 (ab2064, AbCam), anti-IL-10 at 1:100 (JESS-16E3, eBioscience), and anti-pIκB at 1:500 (S32/36, 5A5, Cell Signaling), followed by incubation with biotinylated goat anti-rabbit or goat anti-mouse secondary antibodies at 1:750 using the ABC kit (Vector Labs). Sections were developed using streptavidin-conjugated HRP and substrate, counterstained with hematoxylin, then dehydrated and mounted with Cytoseal 60 (Richard-Allan Scientific). Representative formalin-fixed tissues were stained by hematoxylin and eosin. Images were assessed by light microscopy using an Axio Imager 2 (Zeiss).

Quantitative RT-PCR

Total RNA from frozen prostate tissue was used to synthesize cDNA using High Capacity cDNA Reverse Transcription Kits (Applied Biosystems). Relative gene expression was determined using SYBR Green PCR Master Mix (Applied Biosystems) on a Bio-Rad iCycler, normalized to GAPDH as a gene reference with the comparative threshold cycle method. Primers sets for the following genes were used: Arginase-1, 5′-AGAGATACTTC-CAACTGCCAGACT, 3′-ACCTGGCCTTTGTTGATG-TCCCTA; iNOS, 5′-GCTGGAAGCCACTGACAC-TTCG, 3′-CGAGATGGTCAGGGTCCCCT; GAPDH, 5′-GACCCCTTCATTGACCTCAAC, 3′-CTTCTCCAT-GGTGGTGAAGA.

Flow Cytometry

Spleens were dispersed into single cell suspensions and stained with immune cell markers CD4-APC (RM4–5, BD Bioscience), CD8-FITC (53–6.7, BD Bioscience), B220-FITC (RA3–6B2, BD Bioscience), CD11b-FITC (M1/70, BD Bioscience), GR1-PE (RB6–8C5, BD Bioscience), Foxp3-PE (MF23, BD Bioscience), and NK1.1-PE (PK136, BD Biosciences). For Foxp3 staining, cells were fixed and permeabilized using an intracellular staining protocol per manufacturer's instructions (00–5523-00, eBioscience). Cells were analyzed on an LSRII flow cytometer (BD Biosciences).

RESULTS

More Extensive PIN and Adenocarcinoma in Prostates of MyD88$^{-/-}$ TRAMP$^{Tg+/-}$ Compared to MyD88$^{+/+}$ TRAMP$^{Tg+/-}$ Mice

To examine the role of MyD88 in prostate cancer development, we crossed TRAMP$^{Tg+/-}$ mice with MyD88$^{-/-}$ mice in a C57Bl/6 background to generate a

syngenic immune-competent prostate cancer model. Male MyD88$^{+/+}$ TRAMP$^{Tg-/-}$, MyD88$^{+/+}$ TRAMP$^{Tg+/−}$, MyD88$^{-/-}$ TRAMP$^{Tg-/-}$, and MyD88$^{-/-}$ TRAMP$^{Tg+/−}$ mice were sacrificed at 25 weeks and prostate sections were stained with hematoxylin and eosin and examined by light microscopy. Prostates from non-transgenic MyD88$^{+/+}$ and MyD88$^{-/-}$ mice showed normal prostate development, while comparable development of PIN without areas of adenocarcinoma were observed in prostates of TRAMP$^{Tg+/−}$ MyD88$^{+/+}$ and MyD88$^{-/-}$ animals (Fig. 1A). To better understand the role of MyD88 in development of adenocarcinoma, we elected to examine a cohort of animals at 30 weeks. In the absence of the TRAMP transgene, prostates from MyD88$^{+/+}$ TRAMP$^{Tg-/-}$ and MyD88$^{-/-}$ TRAMP$^{Tg-/-}$ mice showed similar glandular architecture. Interestingly, prostates from MyD88$^{-/-}$ TRAMP$^{Tg+/−}$ mice revealed larger and more densely packed glands then prostates from MyD88$^{+/+}$ TRAMP$^{Tg+/−}$ mice. These glands exhibited higher density of PIN as well as increased areas of well-differentiated adenocarcinoma, defined as loss of p40 staining and disruption of the basal cell layer, in MyD88$^{-/-}$ TRAMP$^{Tg+/−}$ compared to MyD88$^{+/+}$ TRAMP$^{Tg+/−}$ mice (Fig. 1B). In 60% of TRAMP transgenic mice in each genotype, a phyllodes-like tumor was observed arising from the seminal vesicles as previously described with an intact basal layer by p40 expression (Fig. 1C) [24]. Collectively, 30-week prostate and seminal vesicle weights were similar between TRAMP$^{Tg+/−}$ MyD88$^{+/+}$ and MyD88$^{-/-}$ mice (Fig. 1D). To quantitate the extent of PIN and adenocarcinoma, we assessed the percentage of glands harboring PIN and adenocarcinoma and found a significant increase in the prostates from MyD88$^{-/-}$ TRAMP$^{Tg+/−}$ compared to MyD88$^{+/+}$ TRAMP$^{Tg+/−}$ mice (Fig. 1E). The presence of lung metastases were examined histologically and there was no evidence in either MyD88$^{+/+}$ TRAMP$^{Tg+/−}$ or MyD88$^{-/-}$ TRAMP$^{Tg+/−}$ mice (data not shown).

Decreased NK Cells and Increased Myeloid Cells in Tumor Infiltrating Lymphocytes in MyD88$^{-/-}$ TRAMP$^{Tg+/−}$ Mice Compared to MyD88$^{+/+}$ TRAMP$^{Tg+/−}$ Mice

To investigate the role of MyD88 in programming the tumor infiltrating lymphocytes (TILs), we examined expression of distinct immune populations by immunofluorescence staining of prostate tissues. There were no significant differences in expression of the cytotoxic T cell marker CD8 or T regulatory cell marker Foxp3. However, significantly increased myeloid cells, denoted by expression of CD11b and Gr1, and decreased infiltration of NK cells, characterized

by the expression of CD49, were observed in MyD88$^{-/-}$ TRAMP$^{Tg+/−}$ compared to MyD88$^{+/+}$ TRAMP$^{Tg+/−}$ animals (Fig. 2).

CD11b$^+$ Gr1$^+$ Myeloid Cells Represent Myeloid-Derived Suppressor Cells (MDSCs)

To assess the role of MyD88 in mediating tumorigenesis and tumor infiltrating lymphocytes in MyD88$^{-/-}$ TRAMP$^{Tg+/−}$ compared to MyD88$^{+/+}$ TRAMP$^{Tg+/−}$ animals, prostatic expression patterns of MyD88 were examined and found intensely localized in the stroma, along with diffuse expression in the prostate epithelium in MyD88$^{+/+}$ mice (Fig. 3A). Appropriately, no specific staining was found in MyD88$^{-/-}$ mice. MDSCs are early myeloid cells characterized by the surface markers CD11b$^+$ and Gr1$^+$ in mice, and produce cytokines such as IL-10, and arginase-1 and iNOS, to mediate its negative regulatory functions on T cells and NK cells [25]. We examined expression of IL-10 and found increased stromal IL-10 expression in MyD88$^{-/-}$ TRAMP$^{Tg+/−}$ compared to MyD88$^{+/+}$ TRAMP$^{Tg+/−}$ mice (Fig. 3B). Furthermore, whole prostates from MyD88$^{-/-}$ TRAMP$^{Tg+/−}$ mice produced significantly more arginase-1 and iNOS than MyD88$^{+/+}$ TRAMP$^{Tg+/−}$ mice, supporting the expansion of infiltrating MDSCs in MyD88$^{-/-}$ TRAMP$^{Tg+/−}$ mice (Fig. 3C).

Deficiency of NK Cells in Spleens of MyD88$^{-/-}$ TRAMP$^{Tg+/−}$ and MyD88$^{-/-}$ TRAMP$^{Tg-/-}$ Mice

As the absence of MyD88 biased the composition of the tumor microenvironment with respect to tumor infiltrating NK and myeloid cells, we were interested in characterizing the immune populations of spleens from MyD88$^{+/+}$ and MyD88$^{-/-}$ mice as a measure of the systemic influences in immune cell development. In 30-week old mice, no significant differences were observed in the myeloid, B cell, or T cell lineages of the various genotypes. However, a significant decrease in NK cells was observed in both MyD88$^{-/-}$ TRAMP$^{Tg-/-}$ and MyD88$^{-/-}$ TRAMP$^{Tg+/−}$ animals compared to their wild-type counterparts (Fig. 4).

Loss of MyD88 Results in No Significant Differences in NF-κB Signaling or AR Expression

Activation of TLRs through MyD88-dependent pathways leads to activation of canonical NF-κB pathways through the NEMO/IKKα/IKKβ complex resulting in phosphorylation of IκB, allowing translocation of p50/p65 subunits to the nucleus [26]. With a bias composition in the tumor infiltrating lymphocytes and increased areas of prostate adenocarcinoma
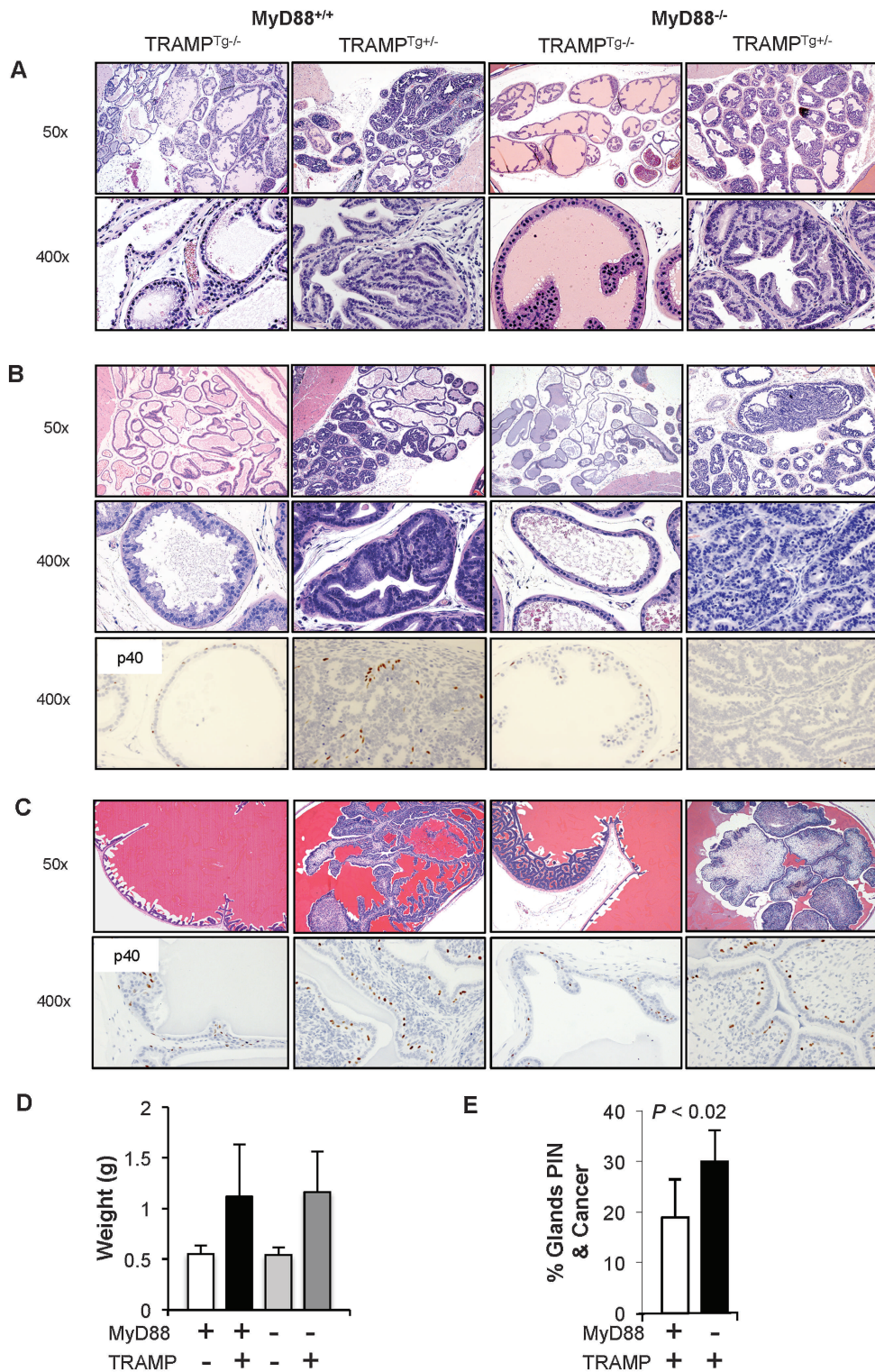
Fig. 1. MyD88^{-/-} TRAMP[Tg+/-] mice show more aggressive prostate adenocarcinoma compared to MyD88^{+/+} TRAMP[Tg+/-] mice. (A) Histology by H&E staining of prostates from mice as indicated at 25 weeks. Histology by H&E staining and immunohistochemistry by p40 staining of prostates (B) and seminal vesicles (C) from 30 week-old mice. (D) Prostate and seminal vesicle weights from mice at 30 weeks. (E) Percentage of glands containing PIN or adenocarcinoma. Magnification as indicated. Columns, mean of five animals; bars, standard deviations. Data are representative of five mice per group.
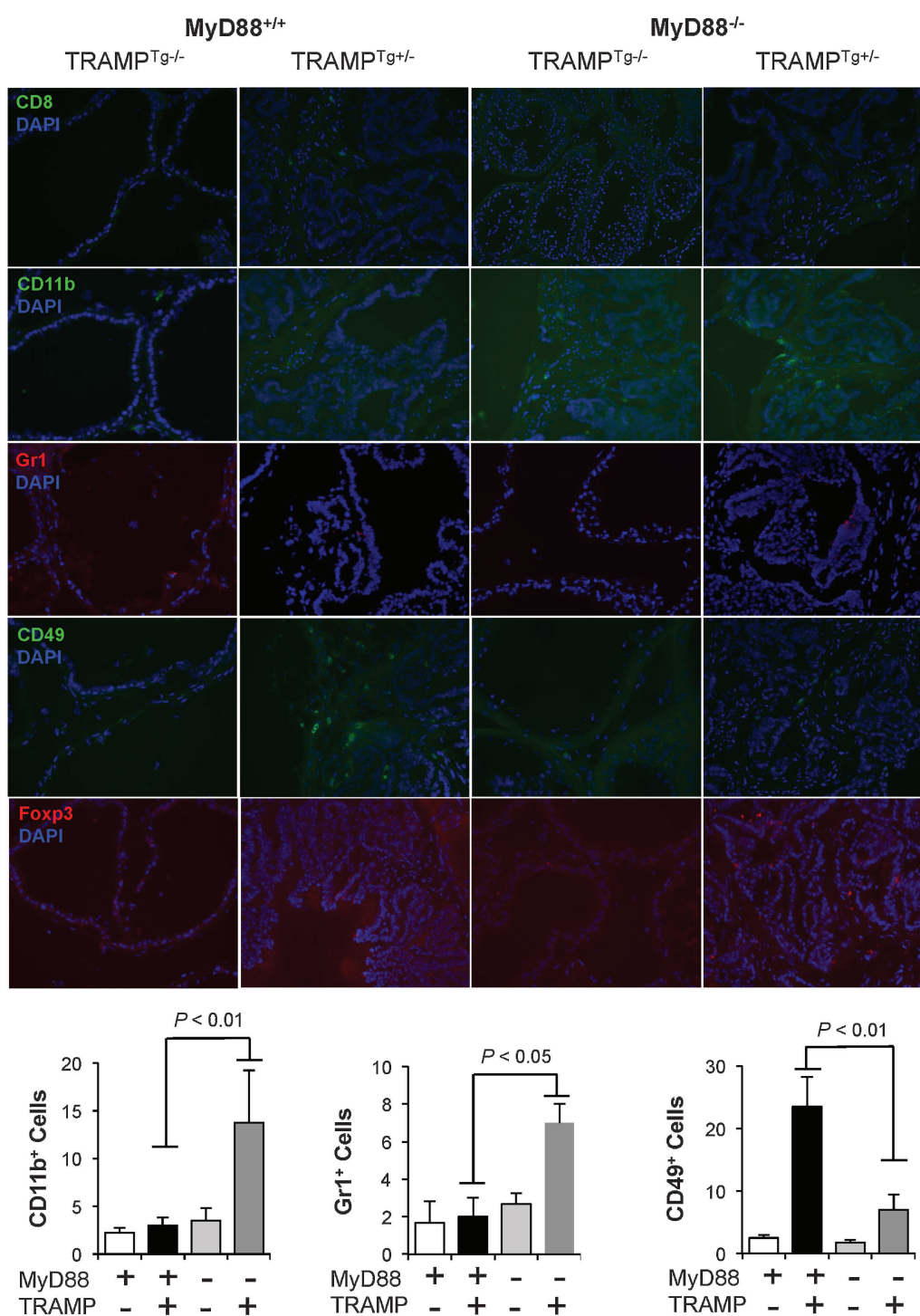
Fig. 2. MyD88^{-/-} TRAMP[Tg+/−] prostates show distinct TIL populations when compared to MyD88^{+/+} TRAMP[Tg+/−] mice. Prostate sections stained by immunofluorescence using immune cell markers as indicated to determine the infiltration of specific immune populations in 30 week MyD88^{+/+} TRAMP^{-/-}, MyD88^{+/+} TRAMP[Tg+/−], MyD88 [-/-] TRAMP^{-/-}, MyD88^{-/-} TRAMP[Tg+/−] mice as indicated. Representative merged fluorescence images are shown (400×). CD11b, Gr1, and CD49 quantified by mean positive staining cells in four high-powered field fields (400×); bars, standard deviations. All P-values were determined by two-tailed Student's t-test, with statistical significance defined as $P < 0.05$.
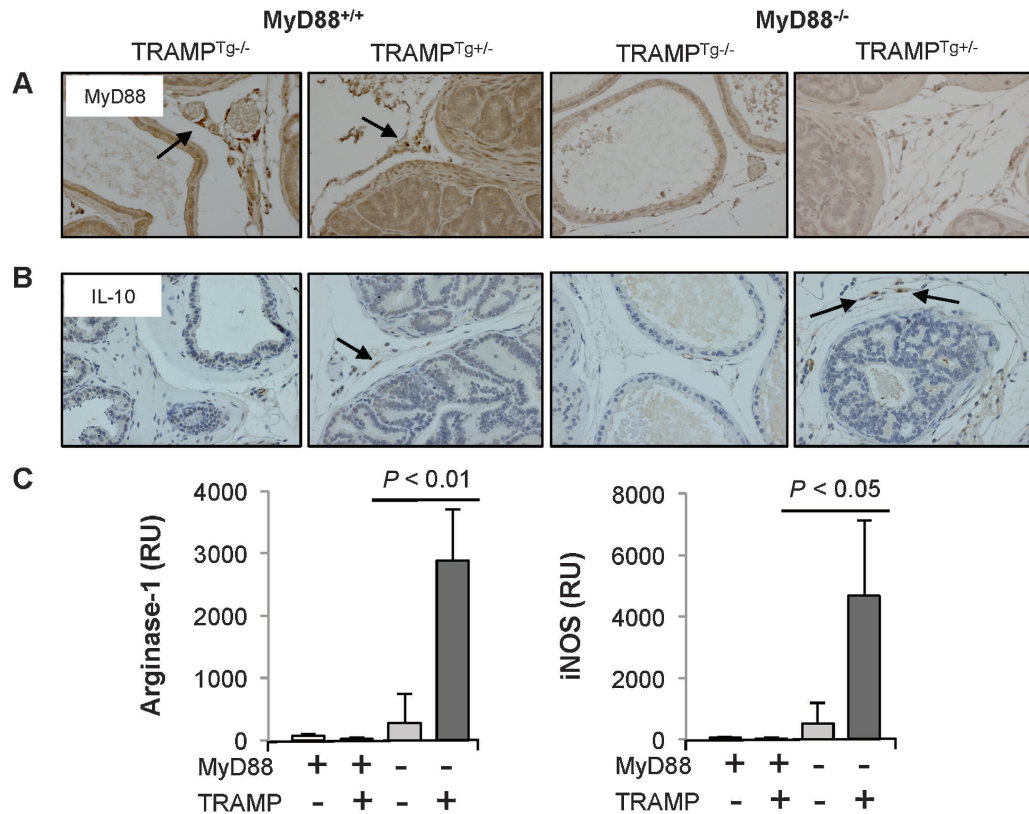
Fig. 3. Increased expression of IL-10, arginase-1, and iNOS from prostates of MyD88^{-/-} TRAMP[Tg+/−] compared to MyD88^{+/+} TRAMP[Tg+/−] mice. Prostate sections stained by immunohistochemistry using antibodies against MyD88 (A) and IL-10 (B). Representative images (400x) shown, with arrows highlighting representative stained cells. (C) Levels of arginase-1 and iNOS by quantitative PCR from prostate tissue of mice as indicated. Columns, mean of three animals; bars, standard deviations. All P-values were determined by two-tailed Student's t test, with statistical significance defined as $P < 0.05$.

in absence of MyD88, we asked whether this phenotype would be associated with altered activation of NF-κB. Staining of prostates from MyD88^{+/+} TRAMP[Tg-/-], MyD88^{+/+} TRAMP[Tg+/−], MyD88^{-/-} TRAMP[Tg-/-], and MyD88^{-/-} TRAMP[Tg+/−] animals revealed similar activation of canonical NF-κB in prostate epithelial cells manifested by detection of phosphorylated IκB (p-IκB) (Fig. 5A). As NF-κB has been linked with androgen receptor expression, we examined expression of AR, which appeared unchanged with the loss of MyD88 [27–30] (Fig. 5B).

DISCUSSION

Using the TRAMP autochthonous prostate cancer model, we have observed increased areas of PIN and adenocarcinoma of the prostate in the absence of MyD88. This result is consistent with our prior observations of PRRs TLR3 and the intracellular kinase Rip2 of Nod-like receptors in tumor surveillance and in programming distinct lymphocyte populations within the tumor microenvironment [15,20]. In each of these instances, the nature of the inflammatory

microenvironment correlated with the response in tumorigenesis. The predominant stromal expression of MyD88 suggests that the prostate epithelium is responding to the altered tumor microenvironment rather than an intrinsic alteration. Similarly, MyD88 blockade has been shown to increase inflammation and progression in a murine model of TLR4-accelerated pancreatic carcinogenesis, thought to be in part mediated by dendritic cell induction of a Th2-polarizing response [31]. However, blockade of TRIF protected against this model of pancreatic carcinogenesis, which is not congruent with our observations of TLR3 signaling in prostate cancer [15]. The anti-tumor effect of TLRs and their signaling molecules is supported by the efficacy of TLR agonists as adjuvants to enhance host immunity with the TLR7 agonist imiquimod FDA approved for treatment of basal cell carcinoma and TLR9 agonists in clinical trials against malignancies including breast, melanoma, and lymphomas [32–36].

Nonetheless, the role of TLRs in tumor surveillance and modulating cancer is not clear, as reports have also supported tumor-promoting effects. For instance, deficiency in MyD88 has been shown to decrease the
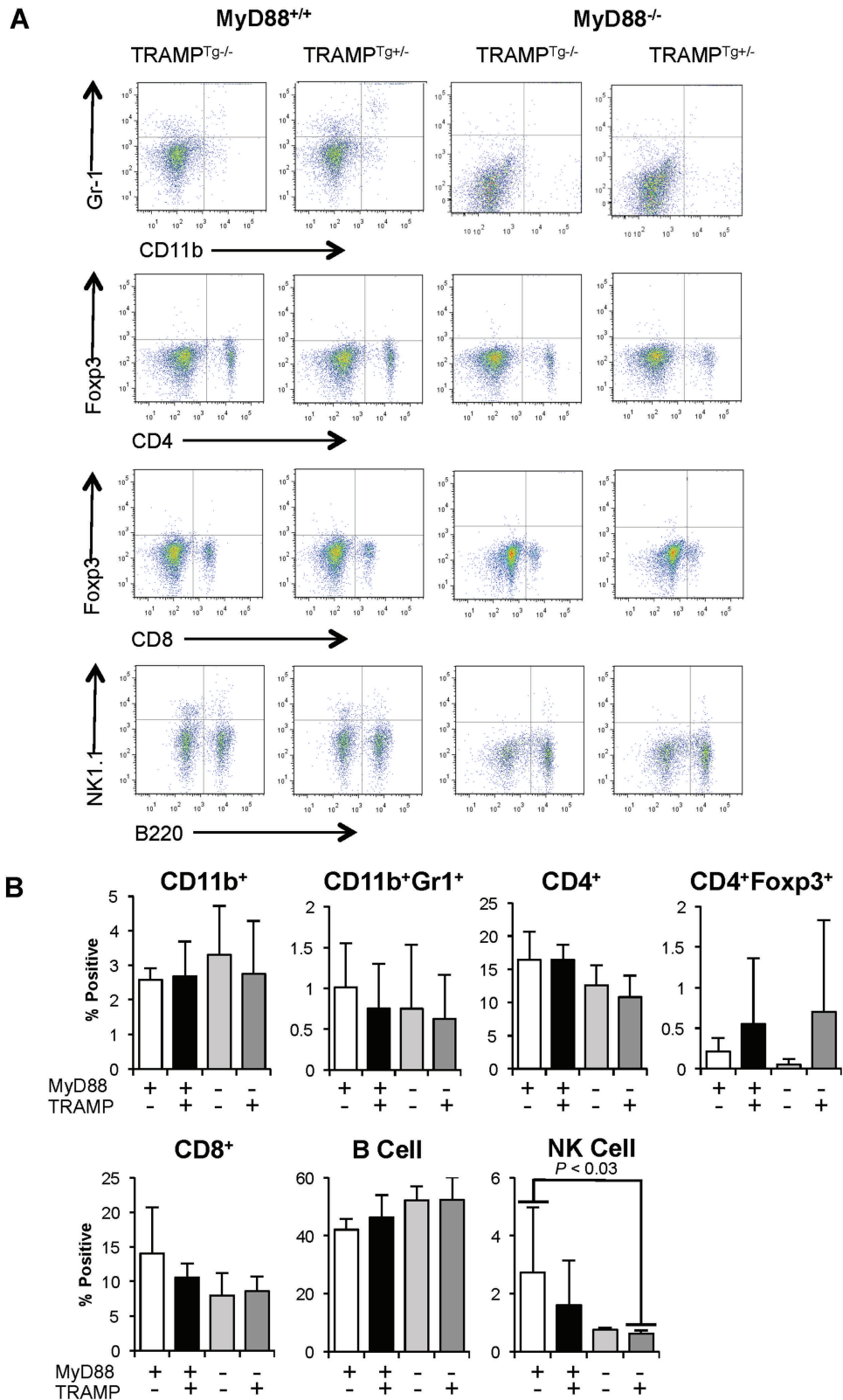
Fig. 4. Splenocytes from MyD88$^{-/-}$ TRAMP$^{-/-}$ and MyD88$^{-/-}$ TRAMP$^{Tg+/-}$ mice show a deficiency in NK cells. (A) Representative flow cytometry of splenic immune populations from mice as indicated. (B) The percentage of immune populations in total splenocytes is shown for each genotype as indicated. Columns, mean of five animals; bars, standard deviations. All p values were determined by two-tailed Student's t-test, with statistical significance defined as $P < 0.05$.
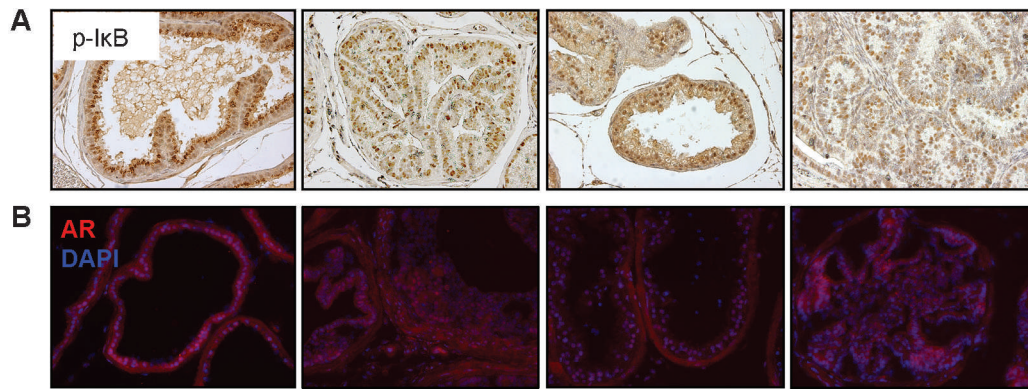
Fig. 5. Prostates of MyD88$^{+/+}$ TRAMP$^{Tg+/-}$ and MyD88$^{-/-}$ TRAMP$^{Tg+/-}$ mice show similar canonical NF-κB activation and AR expression. Representative prostates stained by immunohistochemistry with phospho-IκB and by immunofluorescence with AR, merged fluorescence images shown (400×).

development of tumors in mouse models of spontaneous colorectal cancer and diethylnitrosamine-induced hepatocellular tumors, through mechanisms including enhancing tumor evasion and tissue repair [37,38]. In immune- or tumor-specific knockouts of IKKβ using a colitis-associated cancer model, investigators showed that loss of IKKβ in the tumor epithelium decreased tumor incidence, while loss in myeloid cells led to decreased tumor size [39]. Previously, TLR4$^{-/-}$ TRAMP$^{Tg+/-}$ animals showed a delay in the onset of palpable tumor from 26 to 31 weeks compared to WT controls, however no histology or examination of TILs were performed [40]. This apparent discrepancy may be explained by the pleiotropic TLRs that utilize MyD88 to active NF-κB and MAP kinase pathways, the persistence of MyD88-independent pathways, and the cellular distribution of these receptors in the tumor microenvironment. Perhaps the distribution and bias between epithelial and stromal expression of PRRs and specificity of signaling remains a critical question. Despite these differences in specific knockouts of TLR signaling components, the influence of positive or negative immune regulators and tumor growth remain consistent.

In our study, we identified the decreased presence of tumor infiltrating NK cells and increased CD11b+ Gr1$^+$ cells in the absence of MyD88 in TRAMP murine prostates at 30 weeks (Fig. 3). The loss of infiltrating NK cells in MyD88$^{-/-}$ prostates reinforces previously observed NK-mediated IFN-γ production in response to *Chlamydia trachomatis* infection [41]. Further characterization of the activity of NK cells and the subset of CD11b$^+$ Gr1$^+$ cells will be an important future direction. Although TLRs have been shown to inhibit negative regulatory cells such as Tregs, the relationship between TLRs and myeloid-derived suppressor cells (MDSCs) is less clear [42,43]. Our findings support the MyD88 pathway in modulating infiltrating myeloid-derived suppressor cells, which have been implicated in tumor immune evasion and progression and may explain the decrease in NK cells that we observed [25,44]. It is quite possible that distinct TLR pathways in the context of different tumors and tumor characteristics can specifically shape and program the tumor infiltrating microenvironment. It is unclear the specificity of upstream TLRs utilizing MyD88 in prostate cancer and the bias between MyD88-dependent and -independent pathways upon their activation. We expect future work will categorize the various PRR signaling pathways that will differentially regulate the prostate immune tumor microenvironment.

We observed no significant difference in canonical NF-κB activation comparing prostates of MyD88$^{+/+}$ TRAMP$^{Tg+/-}$ compared to MyD88$^{-/-}$ TRAMP$^{Tg+/-}$ mice. These data suggest that MyD88-independent pathways may exert the majority activation of NF-κB, which has been implicated in development of castrate resistant prostate cancer. A prior report in a subcutaneous model of prostate cancer showed that loss of IKKβ in immune cells prevented metastasis and delayed castration resistance in part through lymphotoxin expression, which can activate non-canonical NF-κB through the LTβ receptor [45,46]. Future directions will specifically examine the mechanistic nature of canonical versus non-canonical NF-κB signaling pathways and their influence in TILs, to examine if a dichotomous relationship exists. Care may need to be exercised in designing therapeutic TLR agonists that will preferentially activate the canonical versus non-canonical pathway in balancing anti-tumor and pro-tumor effects.

One of the criticisms of the TRAMP tumor model is the high percentage of neuroendocrine differentiation

compared to human prostate cancers. In our studies, we did not observe any neuroendocrine differentiation, which appears more common when crossed to the FvB background. An alternative PTEN$^{loxp/loxp}$ x PB-Cre4$^+$ mouse model has been suggested to more closely mimic the human disease [47]. Indeed, prostates from PTEN$^{loxp/loxp}$ x PB-Cre4$^+$ show an expansion of CD11b+ Gr1+ MDSCs [40]. However, the majority of immunological studies have utilized the TRAMP model. Another limitation of our system is that we cannot discriminate loss of MyD88 in the immune system, stroma, or prostate epithelial tissues although expression was highest in the stroma. Future directions will utilize models that can combine different genotypes in the immune, tumor, and stromal environments that will define the role of MyD88 and other PRR signaling components in these distinct compartments. Preliminary studies have shown that a kidney implantation model holds promise in dissecting out the various compartments [48].

CONCLUSIONS

The composition of the tumor microenvironment can alter tumor growth by mediating tumor surveillance and mediating negative immune regulators. We have provided evidence that MyD88 signaling pathways can alter the tumor immune microenvironment and development of prostate cancer. Future studies will need to clarify the mechanisms involved and whether activation of MyD88-dependent pathways can reverse our observations. Defining the role of tumor immune surveillance in the prostate cancer microenvironment will contribute towards the basic comprehension of tumor immunology as well as the development and enhancement of novel therapeutics, vaccines, and immune adjuvants against prostate cancer.

ACKNOWLEDGMENTS

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